Identification of Lutein and Zeaxanthin Oxidation Products in Human and Monkey Retinas

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Purpose. To characterize fully all the major and minor carotenoids and their metabolites in human retina and probe for the presence of the oxidative metabolites of lutein and zeaxanthin.

Methods. Carotenoids of a composite of 58 pairs of human retinas and a monkey retina were elucidated by comparing their high-performance liquid chromatography (HPLC)–ultraviolet/visible absorption spectrophotometry (UV/Vis)-mass spectrometry (MS) profile with those of authentic standards prepared by organic synthesis.

Results. In addition to lutein and zeaxanthin, several oxidation products of these compounds were present in the extracts from human retina. A major carotenoid resulting from direct oxidation of lutein was identified as 3-hydroxy-β,ϵ-caroten-3′-one. Minor carotenoids were identified as: 3′-epilutein, ϵ,ϵ-carotene-3,3′-diol, ϵ,ϵ-carotene-3,3′-dione, 3′-hydroxy-ϵ,ϵ-caroten-3-one, and 2,6-cyclolycopene-1,5-diol. Several of the geometric isomers of lutein and zeaxanthin were also detected at low concentrations. These were as follows: 9-cis-lutein, 9′-cis-lutein, 13-cis-lutein, 13′-cis-lutein, 9-cis-zeaxanthin, and 13-cis-zeaxanthin. Similar results were also obtained from HPLC analysis of a freshly dissected monkey retina.

Conclusions. Lutein, zeaxanthin, 3′-epilutein, and 3-hydroxy-β,ϵ-caroten-3′-one in human retina may be interconverted through a series of oxidation-reduction reactions similar to our earlier proposed metabolic transformation of these compounds in humans. The presence of the direct oxidation product of lutein and 3′-epilutein (metabolite of lutein and zeaxanthin) in human retina suggests that lutein and zeaxanthin may act as antioxidants to protect the macula against short-wavelength visible light. The proposed oxidative-reductive pathways for lutein and zeaxanthin in human retina, may therefore play an important role in prevention of age-related macular degeneration and cataracts. Invest Ophthalmol Vis Sci. 1997:38:1802–1811.

In 1945, Wald[1] tentatively identified the yellow pigment in the human macula as a carotenoid belonging to the xanthophyll families in green leaves. For nearly 40 years, no attempt was made to establish unequivocally the identity of this carotenoid in the human macula, which is still referred to as xanthophyll in many ophthalmology texts. In 1985, for the first time, Bone et al[2] presented preliminary evidence that the human macular pigment is a mixture of lutein and zeaxanthin. A few years later, these pigments, which can be classified as hydroxycarotenoids with no vitamin A activity, were also detected in the human macula and whole retina by Handleman et al.[3] Using high-performance liquid chromatography (HPLC), Bone et al further studied the retinal distribution of lutein and zeaxanthin for 87 donors aged 3 to 95 years.[4] The lutein:zeaxanthin ratio increased in individual retinas from an approximate average of 1:2.4 in the central (0 to 0.25 mm) to more than 2:1 in the periphery (8.7 to 12.2 mm).[4,5]

In 1993, Bone et al[6] elegantly established the complete identification and stereochemistry of the human macular pigment as lutein [(3R,3′R,6′R)-β,ϵ-car-

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otene-3,3′diol], zeaxanthin [(3R,3′R)-β,β-carotene-3,3′diol], and *meso*-zeaxanthin [(3R,3′S)-β,β-carotene-3,3′diol]. In a case–control study published in 1994, high consumption of fruits and vegetables, rich specifically in lutein and zeaxanthin, was correlated with a lower risk for age-related macular degeneration (AMD).[7] The results from this study indicated that the persons consuming green leafy vegetables containing high concentration of lutein (~6 mg/d) had 43% lower risk of exudative AMD relative to the subjects in the lowest quintile.

During the past 13 years, we have reported on detailed qualitative and quantitative distribution of carotenoids in fruits, vegetables, and human plasma. We have shown that although 40 to 50 carotenoids may be present in a typical diet for someone living in the United States,[8,9] only 14 major dietary carotenoids have been identified to date in human plasma.[10–12] Among these plasma carotenoids, we identified four oxidative metabolites of lutein and zeaxanthin and, for the first time, by conducting several metabolic studies involving humans, established the in vivo oxidation of these carotenoids.[13,14]

Several epidemiologic studies suggested that individuals with low plasma concentrations of carotenoids and antioxidant vitamins are at increased risk for AMD.[7,15–17] If the antioxidant efficacy of the most abundant carotenoids in the human retina (i.e. lutein and zeaxanthin) were established, the results from these epidemiologic studies would be easier to interpret. One approach to such investigation would involve complete characterization of the carotenoids (major and minor) in human retina. In addition, to establish the role of carotenoids as antioxidants in prevention of AMD, it is essential to search for the presence of the oxidation products of these compounds in retina, particularly those of lutein and zeaxanthin.

This article reports on identification of three major and 11 minor carotenoids in human retina by HPLC-UV/visible absorption spectrophotometry-mass spectrometry (Vis-MS). Several oxidation products of lutein and zeaxanthin not of dietary origin are among the newly identified carotenoids.

MATERIALS AND METHODS

Source and Sample Preparation

Donor human eyes were obtained from the National Disease Research Interchange (Philadelphia, PA) and the Moran Eye Center (University of Utah, Salt Lake City, UT). They were transported on ice and reached us within 36 hours of death. Procurement methods for tissues used in this study were humane, included proper consent and approval, and complied with the tenets of the Declaration of Helsinki. After the anterior sections were removed, both neural retina and retinal pigment epithelia were frozen at −70°C. Healthy rhesus monkeys (*Macaca mulatta*) 2 to 4 years of age were treated and cared for in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The use of monkey retina was approved by the National Eye Institute's Animal Care and Use Committee and the investigation reported here adhered to the ARVO guidelines for the use, design, and the conduct of experiments involving animals. A pair of freshly dissected monkey retinas was removed immediately after the animal was killed and stored at −70°C until analysis.

Large-Scale Extraction of Human Retina

All extraction, work-up procedures, and analyses were conducted under yellow light to prevent photo-isomerization and degradation of carotenoids. For the preparation of the pool, 58 pairs of retinas were removed from the thawed posterior poles of eyes by dissection, then combined and transferred to a 250 ml beaker. Tetrahydrofuran (THF, 200 ml) containing 0.1% butylated hydroxytoluene (BHT) was added and the retinas were extracted by sonication at 5°C to 10°C for 30 minutes. The mixture was filtered on a Büchner funnel (Whatman Filter Paper No. 113, Fairfield, NJ) and the solid materials were homogenized with THF (100 ml) in a Waring blender at 5°C to 10°C for 15 minutes and filtered as above. The combined filtrate was concentrated using a rotary evaporator at 30°C and the residue (~50 ml) was partitioned between 10% sodium chloride (100 ml) and a mixture of hexane : dichloromethane : methanol : N,N - diisopropylethylamine (DIPEA) at 75%:25%:0.25%:0.1% (100 ml, HPLC eluent A). The organic layer was removed, dried over sodium sulfate, and evaporated to dryness. The residue was filtered through 0.45 μm disposable Acrodisc polyvinylidene fluoride filter assembly (American Scientific Products, McGraw Park, IL) using approximately 5 ml of HPLC eluent A. The solvent was evaporated under nitrogen to dryness and eluent A was added until the total volume of the extract was 3.0 ml; 50 μl samples were injected on HPLC system one.

Extraction of Individual Donor Retinas

Pairs of retinas from individual donors were placed in a 50 ml centrifuge tube and treated with 1 ml of the 3′-ethoxylutein (internal standard) in THF (0.042 μg/ml). The tissues were extracted by sonication using 15 ml of THF (0.1% BHT) at 5°C to 10°C for 15 minutes. The solution was centrifuged at 20,000*g* for 5 minutes; the extract was transferred to a 100 ml round bottom flask. The tissues were reextracted with THF twice (2 × 15 ml) as above. The extracts were combined and evaporated to dryness using a rotary evaporator. The

residue was transferred to a 5 ml vial containing 1 ml of water using HPLC eluent A (~3 ml). The upper organic layer was removed and filtered through 0.45 μm disposable Acrodisc polyvinylidene fluoride filter assembly into a 5-ml graduated micro-sample vial (American Scientific Products, McGraw Park, IL). The water layer was washed with eluent A (1 ml) and the upper layer was removed, filtered as above, and combined with the filtrate in the micro-sample vial. The extract was evaporated to dryness under nitrogen and the residue was reconstituted in 200 μl of eluent A. The vial was centrifuged at 20,000*g* to remove the minor amounts of white solid particles; 50 μl samples were injected on HPLC system two.

Extraction of Fresh Monkey Retina

A pair of monkey retinas (~0.76 g) were extracted according to the procedure described above, and the final extract was reconstituted in 150 μl; a 50 μl sample was injected on HPLC system two.

Chromatographic Systems

Qualitative HPLC separations of carotenoids in a concentrated and combined extract from 58 pairs of human retinas were conducted on HPLC system one, which was equipped with a photodiode array detector and a particle beam mass spectrometer. Because of the low sensitivity of the photodiode array detector, routine and quantitative HPLC separations of carotenoids in extracts from individual retinas were conducted on HPLC system two equipped with a conventional photo-multiplier detector.

High-Performance Liquid Chromatography System One

A Beckman model 114M solvent delivery system equipped with a Beckman model 421 controller (Fullerton, CA) was interfaced into a Hewlett-Packard (HP) 1040A rapid-scanning UV/visible photodiode array detector (Fullerton, CA). The data were stored and processed by a HP 9000/Series 300 (Chem-Station) computing system, in combination with a HP model 9153B disk drive, color display monitor, model 35741, and a model 7470A plotter. The absorption spectra of the carotenoids were recorded between 200 and 600 nm at a rate of 12 spectra/minute. Separations were carried out on a silica-based nitrile bonded (25-cm length × 4.6 mm internal diameter; 5-μm spherical particle) column (Regis Chemical, Morton Grove, IL), which was protected with a Brownlee nitrile bonded guard cartridge (3-cm length × 4.6 mm ID; 5-μm particle size). The eluent consisted of an isocratic mixture of hexane (74.65%), dichloromethane (25.00%), methanol (0.25%), and DIPEA (0.10%). The column flow rate was 0.7 ml/minute. For reproducible separations with this eluent, accurate composition of each solvent, particularly that of methanol, was maintained by preparing this HPLC eluent as needed. This is because of volatility of hexane and dichloromethane that may result in gradual evaporation of these solvents when stored in loosely capped HPLC containers. The monitoring wavelength with this eluent was 445 nm.

This HPLC System was interfaced into a Hewlett-Packard model 5989A particle beam mass spectrometer. The entire eluate from the HPLC system one was allowed to enter the particle beam interface operated at a desolvation temperature of 45°C. Electron capture negative ionization (ECNI) was achieved using methane at a pressure of 0.85 torr and a source temperature of 250°C. Spectra were collected from mass/charge 100 to 700 using a scan cycle time of 1.5 seconds.

High Performance Liquid Chromatography System Two

System two consisted of a Beckman System Gold equipped with a solvent Module 116, programmable detector Module 166 and an autosampler 507 (cooled to 13°C with Haake FX circulatory bath). The data were stored and processed on an IBM Personal computing System/2 model 55SX with a color display monitor. Analytical separations with this System were carried out with the same column and under identical HPLC conditions described above.

Carotenoids Standards and Reagents

The reference sample of lutein was isolated from a crude saponified extract of marigold flowers in a series of sequential solvent washes and extractions followed by crystallization according to a patented procedure.[18] The isolated lutein samples were further purified by preparative HPLC according to our published method to remove the minor quantities (3% to 5%) of zeaxanthin normally present in the extracts from marigold flowers.[19] The (3R,3'R)-zeaxanthin was isolated from Lycium Chinese Mill, a Chinese fruit known as "Guji" and was shown to be identical with a synthetic sample of this compound obtained from Hoffmann LaRoche. The 3-hydroxy-β,ε-caroten-3'-one[10,20] and 3'-epilutein[10] were synthesized from lutein by oxidation with nickel peroxide followed by reduction with sodium borohydride ($NaBH_4$) according to published procedures. Nickel peroxide hydrate (product no. 36,719–2) was purchased from Aldrich Chemical (Milwaukee, WI) and was renewed and activated according to the procedure described by Nakagawa et al.[21] The 3'-Hydroxy-ε,ε-caroten-3-one was synthesized according to our published method.[10] Lactucaxanthin was isolated from a saponified extract of Romaine lettuce and further purified by preparative thin layer chromatography (TLC) followed by HPLC. The geometric isomers of lutein (9Z, 9'Z, 13Z and 13'Z) and zeaxanthin (9Z

and 13Z) were prepared and purified according to our published procedure.[19] The reference sample of 2,6-cyclolycopene-1,5-diol was prepared by oxidation of lycopene with meta-chloroperbenzoic acid followed by acidic hydrolysis.[22] The chemical structures and purity of all the synthetic and isolated samples were further confirmed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy, UV/Vis, and combined HPLC-MS.

Butylated hydroxytoluene and DIPEA were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC-grade solvents, acetonitrile, dichloromethane, hexane, and methanol (Baxter Scientific Division, McGaw Park, IL) were used without further purification.

Preparation of 3′-Ethoxylutein (Internal Standard)

The 3′-ethoxylutein was prepared from lutein similar to the method of Liaaen-Jensen and Hertzberg.[20] Lutein (100 mg, 0.176 mmol) was dissolved in dichloromethane (50 ml) and ethanol (30 ml). Dilute hydrochloric acid in ethanol (100 ml, 0.5% vol/vol) was added dropwise in 10 minutes and the mixture was stirred at room temperature under an atmosphere of nitrogen for 3 hours. Triethylamine (2 ml) was added and the product was partitioned between dichloromethane (100 ml) and 10% NaCl solution (150 ml). The organic layer was separated, dried over sodium sulfate, and evaporated to dryness under reduced pressure. The yellow solid (100 mg) was crystallized from methanol/dichloromethane (8/1) at −60°C. After drying under high vacuum, the yellow crystals of 3′-ethoxylutein (84 mg, 0.141 mmol, 80%) was shown to be pure by HPLC-UV/Vis-MS (eluent A, system one); λ_{max} (HPLC eluent A) = 270, 334, 424, 448 (main maximum), 476 nm; λ_{max} (dichloromethane) = 336, 431.5, 454.5 ($E^{1\%}$ = 2233), 483.5 nm; MS (ECNI, methane): molecular parent anion peak at m/z = 596 (100%) and an ion peak at m/z = 550 (9%) because of the loss of ethanol from the molecular parent ion peak.

Stability Studies With Lutein, 3′-Epilutein, and Zeaxanthin

Pure samples of lutein, zeaxanthin, and 3′-epilutein were kept in both crystalline form and also in THF solutions exposed to air and yellow laboratory light for 3 days. At the end of this period, the standards were examined by HPLC-UV/Vis-MS to determine photodegradation and isomerization of these compounds.

In a control experiment, a mixture of lutein and zeaxanthin in THF was subjected to all the extraction steps of human retina described earlier and the product was monitored by HPLC to determine the stability of these carotenoids.

RESULTS

Nomenclature

For convenience, the trivial names of certain carotenoids have been used throughout this text. The trivial and correct systematic names for these carotenoids are presented in Table 1. For in-chain geometrical isomers of carotenoids, the terms *all*-E and Z, which refer to *all*-trans and cis isomers of carotenoids, respectively, should be used instead of the old nomenclature. However, because many readers are more familiar with the old nomenclature, we have used the terms *all*-trans and cis throughout this text. The R and S symbols refer to those carotenoids with known configurations. For several of the oxidative metabolites of lutein and zeaxanthin, the R and S symbols were not used because the absolute configurations of these carotenoids with two or more centers of chirality are not known at present.

Identification of Carotenoids in Human and Monkey Retinas

The major carotenoids have been identified as lutein, zeaxanthin, and a direct oxidation product of lutein, namely 3-hydroxy-β,ϵ-caroten-3′-one. Several oxidation products of lutein and zeaxanthin and one of lycopene were also among the minor carotenoids. In addition, the most common geometric isomers of lutein and zeaxanthin, (i.e. 9-cis-lutein, 9′-cis-lutein, 13-cis-lutein, 13′-cis-lutein, 9-cis-zeaxanthin, and 13-cis-zeaxanthin) which are usually present in serum were also detected at low concentrations in retina. Similar results were also obtained from an extract of freshly dissected monkey retina. A typical HPLC profile of a combined extract from 58 pairs of human retina is shown in Figure 1. The retinas were combined to obtain sufficient quantity of the minor carotenoids for detection and identification by HPLC-UV/Vis-MS. The identity of carotenoids, the UV/visible absorption and mass spectral data are shown in Table 1. These were identified by comparison of their HPLC retention times and UV/Vis and MS data with those of synthetic or isolated reference samples of carotenoids.

As shown in Figure 1, in addition to lutein (peak 7) and zeaxanthin [mixture of 3R,3′R- and 3R,3′S,meso-zeaxanthin (peak 8)], which were previously identified in human retinas,[6] a number of carotenoid oxidation products (peaks 1 through 5, and 9) were also present. Lactucaxanthin (Fig. 1, peak 6), a dihydroxycarotenoid found in Romaine lettuce (*Lactuca sativa*), was also detected at a low concentration in human retinas. It is imperative to point out that the HPLC separation de-

TABLE 1. High-Peformance Liquid Chromatography Peak Identification of Carotenoids in Human Retina From Wavelengths of Absorption Maxima and Mass Spectral Data

Peak	*Carotenoids**	*Absorption Maxima†* *(nm)*	*Molecular Mass‡* *(m/z)*
1	ϵ,ϵ-Carotene-3,3′-dione	420,442,472	564
2	3′-Hydroxy-ϵ,ϵ-caroten-3-one	422, 442, 472	566, 548(M-H_2O)
3	2,6-Cyclolycopene-1,5-diol	434, 458, 492	570
4	3-Hydroxy-β,ϵ-caroten-3′-one	(424), 448, 476	566, 548(M-H_2O)
5	(cis)-3-Hydroxy-β,ϵ-caroten-3′-one	(420), 442, 470	566, 548(M-H_2O)
6	ϵ,ϵ-Carotene-3,3′-diol (lactucaxanthin)	416, 442, 470	568, 550(M-H_2O)
7	(*all*-trans,3R,3′R,6′R)-β,ϵ-carotene-3,3′-diol [(*all*-trans,3R,3′R,6′R)-lutein]	(424), 448, 476	568, 550(M-H_2O)
8	(*all*-trans)-β,β-carotene-3,3′-diol [(*all*-trans,3R,3′R)-zeaxanthin + (all-trans,3R,3′S,meso)-zeaxanthin]	(428), 454, 482	568
9	(*all*-trans,3R,3′S,6′R)-β,ϵ-carotene-3,3′-diol [(*all*-trans)-3′-epilutein]	(424), 448, 476	568, 550(M-H_2O)
10	(9-cis,3R,3′R,6′R)-Lutein	334, (420), 442, 470	—
11	(9′-cis,3R,3′R,6′R)-Lutein	332, (420), 444, 472	—
12	(13-cis)-Lutein+(13′-cis)-lutein	334, (418), 440, 468	—
13	(9-cis)-Zeaxanthin	340, (424), 450, 474	—
14	(13-cis)-Zeaxanthin	338, (419), 446, 472	—

Determined by HPLC photodiode array detection–mass spectrometry in the order of elution on a nitrile bonded column.
* Common names for certain carotenoids are shown in parentheses.
† Values in parentheses represent points of inflection.
‡ The molecular ions appeared as the base peak (100% intensity). In some cases, ions due to the loss of H_2O from the molecular parent ion (M) could also be observed.
Because of the low concentration, the molecular parent ion was not observed.

scribed here did not resolve (3R,3′R)-zeaxanthin and (3R,3′S,meso)-zeaxanthin, and both of these compounds coeluted as peak 8 (Table 1). Therefore, throughout this text, unless specified, the term zeaxanthin refers to the mixture of both (3R,3′S,meso)-zeaxanthin and dietary (3R,3′R)-zeaxanthin. Among the minor carotenoids, the presence of several geometric isomers of lutein and zeaxanthin, identified as 9-cis-lutein, 9′-cis-lutein, 13-cis-lutein, 13′-cis-lutein, 9-cis-zeaxanthin, and 13-cis-zeaxanthin, is of particular interest. This is because these isomers may be the products of in vivo photoisomerization of all-trans-lutein and all-trans-zeaxanthin.

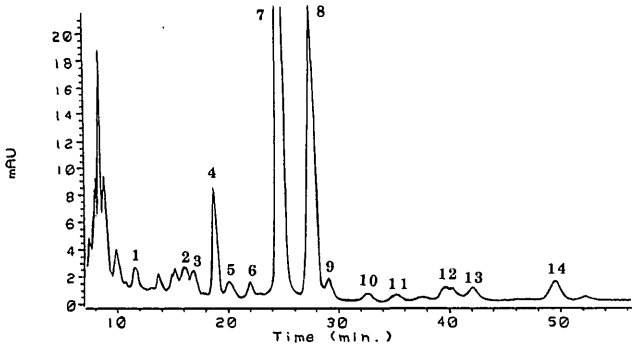

FIGURE 1. High-performance liquid chromatography (HPLC) profile of a combined extract from 58 pairs of human retinas on system one equipped with a photodiode array detector and a particle beam mass spectrometer. The HPLC peaks of (3R,3′R)-zeaxanthin and (3R,3′S, meso)-zeaxanthin are not resolved and both appear as peak 8. Conditions described in text. For peak identification see Table 1.

The chemical structures of carotenoids and their oxidation products are shown in Figure 2. Compounds 1, 2, and 4 are the products of oxidation of all-trans-lutein and all-trans-zeaxanthin and compound 3 was recently identified as a metabolite of lycopene,[14] the red pigment found in tomatoes and tomato-based products. The 3′-epilutein (compound 9) may also be formed by a sequential oxidation and reduction of all-trans-lutein or double-bond isomerization of all-trans-zeaxanthin. The typical carotenoid profiles obtained using a sensitive detector on HPLC system two of retina extracts from individual donor eyes and that of a monkey are compared in Figures 3a and 3b, respectively. In these cases, because of the insufficient quantity of sample, the HPLC peak identification was accomplished from retention times of various peaks and their chromatographic coelution with known synthetic standards. Although several of the minor carotenoids identified in the combined extracts from 58 pairs of human retinas were not detected in individual human and monkey retinas because of low concentrations, the general carotenoid profile of all the retinas was similar.

The qualitative and quantitative distribution of ca-

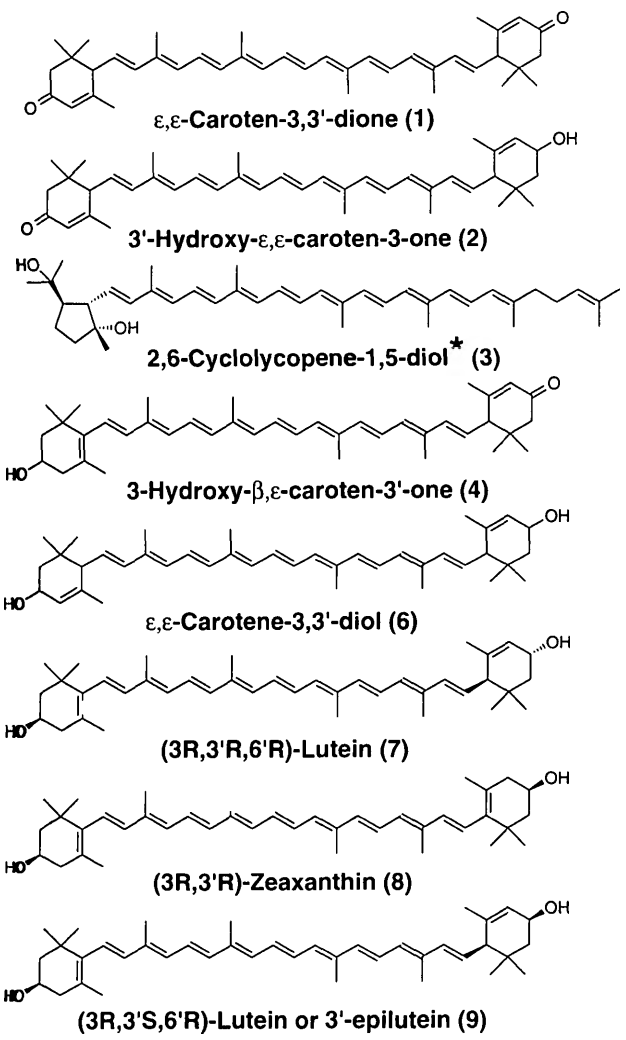

FIGURE 2. Chemical structures of carotenoids and their metabolites identified in human retina. The planar structures for compounds 1, 2, 4, and 6 are shown since the absolute configuration of these carotenoids with two or more centers of chirality is not known. (*) Only the relative but not absolute configuration for 2,6-cyclolycopene-1,5-diol at C-2, C-5, and C-6 is known.

rotenoids in 11 human retinas from donors aged 28 to 89 years and that of a 3-year-old monkey are shown in Table 2. The recoveries of the internal standard, 3'-ethoxylutein, from various extractions of individual human retinas were greater than 95% as determined from the HPLC peak area of this compound before and after extraction. The carotenoid profiles of all the human retinas and that of one monkey showed striking resemblance in that they all contained moderate concentrations of a direct oxidation product of lutein, namely 3-hydroxy-β,ε-caroten-3'-one (peak 4, Figs. 1 and 3, Table 1); this compound was accompanied by minute quantity of a cis-isomer. Other minor components routinely detected in all of the retinas examined, were 3'-epilutein (peak 9, Figs. 1 and 3, Table 1) and the cis-isomers of lutein and zeaxanthin (peaks 10–14, Figs. 1 and 3, Table 1).

Stability Studies With Carotenoids

All-trans-lutein, all-trans-zeaxanthin, and all-trans-3'-epilutein (peaks 7, 8, 9 respectively, Fig. 1, Table 1) were found to be stable and did not undergo stereoisomerization or oxidation under the conditions employed for the extraction of retina. Similarly, exposure of the pure samples of these carotenoids both in crystalline form and in THF solutions to air and yellow laboratory lights after 3 days did not result in the conversion of these compounds to the oxidation products identified in retina. However, after 3 days, approximately 10% of these carotenoids in both crystalline form and THF solutions underwent in-chain oxidative cleavage and degradation to give a number of carotenoid aldehydes known as apocarotenals. These apocarotenals were not detected in any of the human or the monkey retina examined in the current study.

DISCUSSION

Among all the carotenoids identified in human retina, only (all-trans,3R,3'R,6'R)-lutein, (all-trans,3R,3'R)-

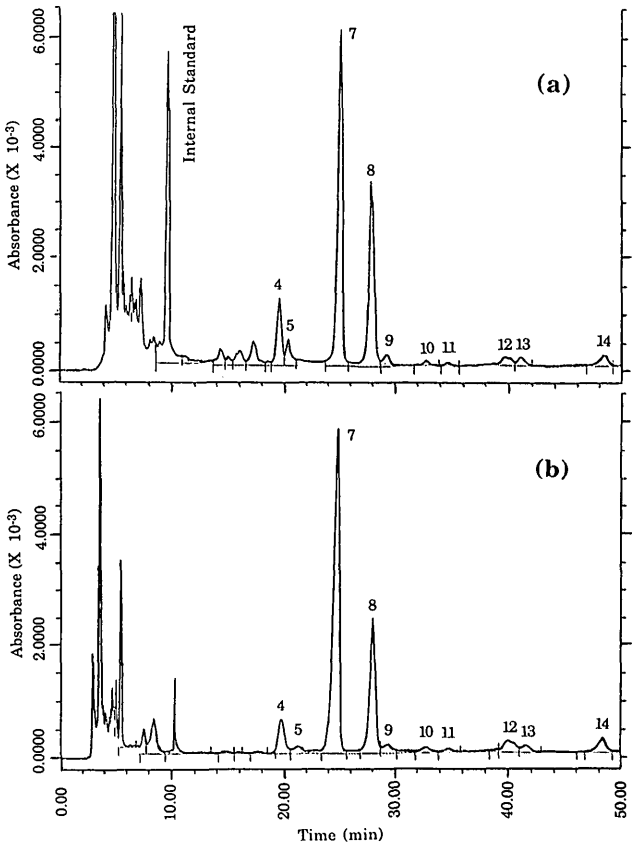

FIGURE 3. High-performance liquid chromatography (HPLC) profiles of retina extracts on system two. (**A**) a pair of retinas from one human subject. (**B**) a pair of retinas from a monkey. Conditions described in text. HPLC peak identification (Table 1) was based on co-chromatography with synthetic standards.

TABLE 2. Carotenoids and Their Oxidation Products Per Two Retinas From 11 Humans and One Monkey for Comparison

		Human Subjects (years of age)										
	Monkey (1) (3 years)	*1 (28)*	*2 (36)*	*3 (38)*	*4 (41)*	*5 (46)*	*6 (50)*	*7 (54)*	*8 (59)*	*9 (61)*	*10 (67)*	*11 (89)*
Carotenoids (peaks)	*(ng)*	*Concentration (ng/2 retinas)*										
all-trans-lutein (7)	37.2	84.5	37.6	22.7	52.3	24.8	199	62.2	236	112	388	58.7
Total cis-luteins (10-12)	4.6	5.9	3.8	2.4	5.5	3.0	35.0	6.3	13.6	10.5	36.3	5.6
all-trans-lutein + cis-luteins	41.8	90.4	41.4	25.1	57.8	27.8	234	68.5	250	123	424	64.3
all-trans-zeaxanthin (8)	21.7	96.3	31.9	23.3	48.3	15.2	117	49.7	155	75.3	254	35.4
Total cis-zeaxanthins (13-14)	4.9	15.0	5.2	3.0	5.2	4.0	35.3	10.5	24.9	7.2	46.1	8.8
all-trans-zeaxanthin + cis-zeaxanthins	26.6	111	37.1	26.3	53.5	19.2	152	60.2	180	82.5	300	44.2
all-trans + cis-3-hydroxy-β,ϵ-caroten-3′-one (4-5)	5.6	21.6	11.3	9.8	17.7	8.1	96.2	15.6	63.8	53.9	94.9	28.4
all-trans-3′-epilutein (9)	0.7	5.6	1.5	2.6	2.6	1.3	15.5	3.7	7.7	6.5	20.7	5.2
		Ratio of Concentrations										
all-trans lutein/total cis-luteins	8.0	14.3	9.9	9.5	9.5	8.3	5.7	9.9	17.4	10.7	10.7	10.5
all-trans zeaxanthin/total cis-zeaxanthins	4.4	6.4	6.1	7.8	9.3	3.8	3.3	4.7	6.2	10.5	5.5	4.0
Total-lutein/total-zeaxanthin	1.6	0.8	1.1	1.0	1.1	1.5	1.5	1.1	1.4	1.5	1.4	1.5

zeaxanthin, all-trans-lactucaxanthin, and their cis-geometric isomers are of dietary origin. Although (3R,3′R,6′R)-lutein is the most abundant carotenoid in all green and some yellow fruits and vegetables, the dietary sources of (3R,3′R)-zeaxanthin are limited to corn, peaches, certain varieties of squash, and citrus fruits.[8] Similarly, among all the commonly consumed fruits and vegetables in the U.S., only Romaine lettuce had moderate concentrations of lactucaxanthin (unpublished results, 1995).

The presence of the oxidation products of lutein and zeaxanthin in human retinas (peaks 1 through 5 and 9, Fig. 1) was at first treated with a great deal of caution because in some cases the retina samples did not reach the laboratory until 36 hours after death. Although upon collection, these samples were stored on ice, there was always the possibility that oxidation of lutein and zeaxanthin may have occurred in retina after the removal of the eyes. However, when a fresh pair of retina from a 3-year-old Rhesus monkey was extracted and analyzed by HPLC, the presence of a direct oxidation product of lutein, namely 3-hydroxy-β,ϵ-caroten-3′-one (compound 4, Fig. 2) and 3′-epilutein (compound 9, Fig. 2) was established unequivocally. In addition, the results from stability studies have clearly indicated that the oxidation products of lutein and zeaxanthin identified in the human and monkey retina are not artifacts of storage, handling, and extraction.

These carotenoid oxidation products are not of dietary origin and have previously been identified in human plasma and breastmilk by Khachik et al.[10–12] It is not known if these carotenoid oxidation products are transported to and accumulated in retina via the circulatory system or whether photo-induced metabolic oxidation of lutein and zeaxanthin may be responsible for their presence. It is important to note that all of these oxidation products are present in both human plasma and retinas at very low concentrations. However, only the direct oxidation product of lutein, 3-hydroxy-β,ϵ-caroten-3′-one, is found in the retina at relatively high concentrations compared with other

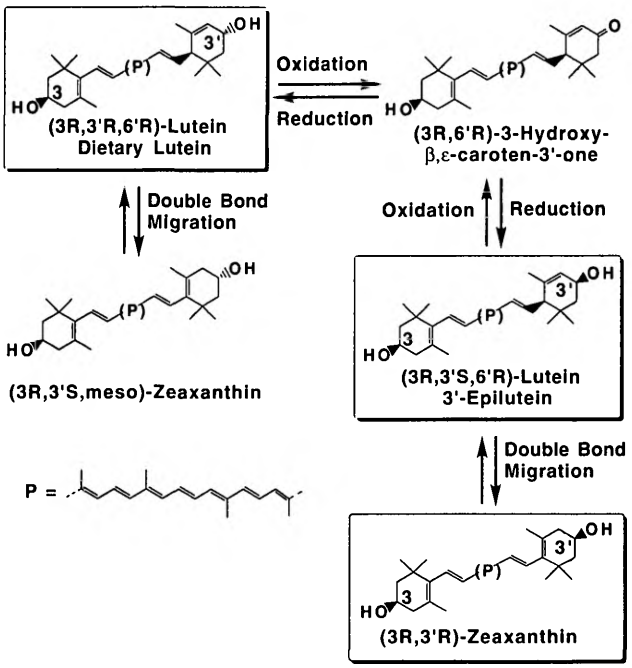

FIGURE 4. Proposed metabolic transformations of dietary (3R,3'R,6'R)-lutein and (3R,3'R)-zeaxanthin in human retina.

oxidative metabolites of carotenoids. Currently, this finding provides the only evidence for a possible in vivo metabolic oxidation of lutein in human retina. The protection of retina from short-wavelength visible light by lutein and zeaxanthin is based on two assumptions. First, the oxidation products of lutein and zeaxanthin are formed in vivo in the retina. Second, these oxidative metabolites are formed by the action of blue light. Based on several supplementation studies with purified lutein and zeaxanthin involving human subjects, we proposed metabolic pathways for conversion of these dietary carotenoids to their oxidation products.[13,14]

According to these metabolic transformations, (3R,3'S,meso)-zeaxanthin, (3R,3'R,6'R)-lutein, and (3R,3'R)-zeaxanthin may be inter-converted through a series of oxidation-reduction and double bond isomerization reactions, as shown in Figure 4. The driving force for the direct oxidation of lutein to 3-hydroxy-β,ϵ-caroten-3'-one is the activation of the hydroxyl group at C-3' by the neighboring allylic double bond. However, in (3R,3'R)-zeaxanthin, because of the non-allylic nature of the hydroxyl groups at C-3 and C-3', this compound is not directly oxidized and may therefore undergo double bond isomerization to yield 3'-epilutein [(3R,3'S,6'R)-lutein] before oxidation. As a result, 3-hydroxy-β,ϵ-caroten-3'-one can also be formed from allylic oxidation of 3'-epilutein. To establish inter-conversion between carotenoids in these metabolic transformations, once 3-hydroxy-β,ϵ-caroten-3'-one is formed it may undergo reduction reactions with epimerization at C-3' to yield lutein and/or 3'-epilutein. In another double bond isomerization reaction, dietary (3R,3'R,6'R)-lutein may be transformed into (3R,3'R,*meso*)-zeaxanthin (Fig. 4). The presence of (3R,3'S,*meso*)-zeaxanthin and the stereochemistry of the macular carotenoids in humans has been elegantly established by Bone et al.[6]

We have determined recently that 98% of zeaxanthin in human plasma is in the form of dietary (3R,3'R)-zeaxanthin and only 2% exists as (3R,3'R,*meso*)-zeaxanthin (unpublished results, 1995). Therefore, the fact that Bone et al[6] have demonstrated that almost equal amounts of (3R,3'R)-zeaxanthin and (3R,3'R,*meso*)-zeaxanthin are present in the human macula indicates that the latter compound may be formed from double bond isomerization of dietary (3R,3'R,6'R)-lutein as shown in Figure 4. Unfortunately, the only evidence in support of these metabolic transformations is the mere presence of 3-hdyroxy-β,ϵ-caroten-3'-one, 3'-epilutein, and meso-zeaxanthin in the retina. The transport and the metabolic interconversions between lutein and the two forms of zeaxanthin in human retinas are most probably induced by the radiation from short-wavelength visible light, catalyzed by certain proteins, or both. It is known that human macular carotenoids bind to retinal tubulin,[23] and other more specific human macular carotenoid binding proteins are being identified and characterized (Bernstein et al, unpublished results, 1996).

The concentrations of the cis/trans stereoisomers of lutein, zeaxanthin, and several of their prominent oxidation products in retinas from 11 human subjects and in one monkey donor are shown in Table 2. From these data, it appears that the concentration of carotenoids and their metabolites vary over a wide range for all subjects regardless of age group. Particularly noticeable is the relatively high concentrations of the total 3-hydroxy-β,ϵ-caroten-3'-one (trans + cis), which range from 8 ng to 96 ng and appear to increase nearly proportionally with lutein concentrations. The ratio of concentration of the total lutein (trans + cis) to total zeaxanthin (trans + cis) in the whole retina varies from 0.8 to 1.5, but it is generally in favor of lutein for nearly all subjects. However, it must be noted that Bone et al[4,5] demonstrated that, in individual retinas, the lutein:zeaxanthin ratio increased from an average of approximately 1:2.4 in the central 0 mm to 0.25 mm to over 2:1 in the periphery (8.7 mm to 12.2 mm). On the other hand, depending on the diet of an individual, the concentration of lutein (trans + cis) in plasma may be as high as 9 times that of zeaxanthin (trans + cis).[12] This is reflective of the much lower dietary distribution of (3R,3'R)-zeaxanthin relative to (3R,3'R,6'R)-lutein.

However, by considering that almost 50% of total zeaxanthin in the human retina is in the form of (3R,3'S,*meso*)-zeaxanthin,[6] which is not dietary and

presumably formed from metabolic transformation of dietary (3R,3'R,6'R)-lutein, one may explain the unusual difference between the ratio of lutein:zeaxanthin in plasma versus retina. It is important to note that the quantitative data presented in Table 2 are from pairs of retinas pooled from individual donors and therefore these concentrations do not account for pigment variation between left and right eye. Bone et al demonstrated that pigment variations between left and right eye may range from 0 to 43% and averaging 13 ± SD 10%.[4]

As shown in Table 2, the cis-isomers of lutein and zeaxanthin are also present in the human retina at low concentrations relative to their all-trans compounds. It is likely that these cis-isomers may be formed as a result of in vivo photo-induced stereo-isomerization of all-trans-lutein and all-trans-zeaxanthin in the retina because this is one of the most common reactions observed with carotenoids. Another likely source of the cis-isomers of lutein and zeaxanthin may be human plasma where we have previously identified the geometric isomers of these compounds.[10-12,19]

CONCLUSION

The detection of the oxidation products of lutein and zeaxanthin in the human retina supports the hypothesis that dietary (3R,3'R,6'R)-lutein and (3R,3'R)-zeaxanthin may act as antioxidants to protect the retina from over exposure to the short-wavelength visible light. Lutein and zeaxanthin were also recently identified in the human lenses and may also play a role in prevention of cataracts.[24] Therefore, the presence of these compounds in the retina and lens at reasonably high concentrations may be essential to prevent macular degeneration and cataracts. This hypothesis is further supported by the established role of carotenoids in green plants; where lutein is the most abundant component and, along with other carotenoids, prevents the destruction of chlorophylls from over exposure to sunlight. In addition, among the 14 dietary carotenoids routinely detected in human plasma, only lutein and zeaxanthin appear to accumulate in the human retina at much higher concentrations than other equally prominent carotenoids such as lycopene, α-carotene, and β-carotene.

One explanation may be that there are certain structural requirements such as the presence of the two hydroxyl groups in lutein and zeaxanthin, because these compounds are the only two dihydroxycarotenoids in commonly consumed fruits and vegetables. According to a recently published epidemiologic study, high consumption of fruits and vegetables specifically rich in lutein and zeaxanthin may lower the risk for macular degeneration.[7] Clinical intervention trials with purified supplements of lutein and zeaxanthin involving patients at an early stage of macular degeneration will be necessary to determine the efficacy and effectiveness of these compounds in maintenance and treatment of this disease. Currently, lutein is routinely isolated and purified commercially from extracts of marigold flowers (*Tagetes erecta*) according to a U.S. Patent[18] and is readily available whereas the large scale isolation and production of zeaxanthin from natural sources is still under development.

Key words

carotenoid oxidation products, 3'-epilutein, high-performance liquid chromatography–mass spectrometry, 3-hydroxy-β,ϵ-caroten-3'-one, lutein, new carotenoid metabolites, zeaxanthin

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